CATALASE ACTIVITY AS A MEASURE OF SEED VIABILITY.

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Edmonton, Alberta.

April, 1928.

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A THESIS

submitted to the University of Alberta
(Department of Field Crops)
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE.

April, 1928.

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INTRODUCTION.

It is a truism to say that agricultural crop production per unit of land stands on the whole at a much higher level today than was the case in the past. Among the many factors which have contributed to this result not the least important is the use of good seed. Not only should such seed be free from the seeds of undesired species, whether cultivated or not, but it should also have a high degree of vigour and viability, thus giving the seedlings the best possible chance of establishing themselves amid the competition of weeds and other plants. While this has been recognized for a long time it was only at about the beginning of the present century that the systematic testing of seeds began to assume any very considerable importance. From that time forward the establishment of seed testing laboratories became general in all civilized countries.

As a result of this general testing of seed it was found that the stock commonly used was in a deplorable condition and most countries adopted some sort of government supervision and control of the sale of seeds.

In making a test of the vitality of seed, a given number, usually two or more replicates of 100 seeds each, are counted out and placed on suitable media such as soil, sand, or moist blotting paper and left for varying lengths of time to germinate. The percentage of germination after all the seeds remaining undecayed have sprouted is considered a measure of the viability of the seed, while the percentage of germination after a shorter, arbitrary time determined by experience, is considered a measure of the vitality. The usual period of time required for a germination test is several days while some seeds require as long as a month or more to show their germinating capacity. Moreover, in some species the seeds exhibit a dormant condition which results in delayed germination or even makes the seed extremely difficult to germinate in laboratory practise.

It soon became apparent that the long period of time required was apt at times to be a serious hindrance

to trade. Accordingly investigators turned their attention to the problem of securing a satisfactory index of seed viability without the necessity of having recourse to the germination test.

REVIEW OF LITERATURE ON CATALASE AND OTHER BIOCHEMICAL TESTS.

In 1901, Waller (27) published the results of his experiments in which he used an electrical method. He observed that the momentary excitation of living tissue by an electric current produced an after current which he termed a "blaze current". The blaze current was generally in the same direction as the exciting, but in the case of a tissue which gave a blaze current of the contrary sign, the blaze current remained in the same direction when the sign of the exciting current was changed. The blaze current was hence distinguishable from a polarization current. With non-living tissue however, the after current was invariably in the opposite direction to its exciting current and hence was only a polarization effect.

He found that with the soaked radicles of bean seeds (Phaseolus vulgaris) there was a marked negative correlation between the blaze current and the age of the seeds, radicles from dead seeds giving no blaze effects. He found also a correlation between the blaze currents given by whole seeds and their subsequent behaviour in the germination chamber.

He concluded that the blaze reaction was of a physiological character. There was shewn to be a general, but not faultless correspondence as regards magnitude between the blaze reaction and the germination, which was of the order of 0.0500 volts for fresh and vigorous seeds, 0.0100 volts for older and less vigorous seeds, while still older seeds, incapable of germination manifested blaze currents of only 0.0010 volts down to the small counter effect due to polarization of 0.0005 volts more or less. The method appeared suitable where there was a question of great differences of vitality, but less so where the differences were smaller.

Duvel (7) studied enzymes in their relation to the vitality of seeds. He pointed out that the artificial

use of enzymes had greatly increased the germination of some old seeds, but shewed this to be the case only when vitality was at a low ebb and not when extinct. He concluded that the loss of vitality in seeds is not due to the disorganization of the enzymes but that there is a close connection between the two. In some of his experiments he reduced the vitality of seeds by prolonged storage (up to 85 days) in closed bottles with varying quantities of water, which however was not in contact with the seeds but added on small strips of blotting paper. The enzyme he studied was diastase (amylase).

Darsie, Elliott and Pierce (5) based a method for determining the vitality of seeds on the temperatures developed by a given weight of seeds placed in silvered Dewar flasks under conditions suitable for germination. They found a "normal temperature" for each of a number of different kinds of seeds. This normal temperature was the average increase in temperature produced by ten grams of vigorous, high germinating seed under the above conditions, per day. This varied from 1.82°C for hemp to 0.49°C for corn. A temperature in excess of normal was taken to indicate a condition of infection and was generally due to the heat generated by the growth of moulds &c; while a subnormal temperature indicated decreased vigour. Apparently there was no attempt to correlate different temperatures with different percentages of germination.

Lesage (14) in a recent paper, cites his earlier work published in 1911 and 1917 on the determination of viability by methods other than direct germination. He used 20 solutions of KOH varying in concentration from N to $\frac{3}{4}N \times 2^{-9}$, in which he soaked the seeds being tested. He found that seeds having lost their viability coloured all solutions an egg-yolk yellow, while those still viable coloured only the stronger solutions. He worked chiefly with Lepidium sativum, but found his method applicable to eighteen other species.

Appleman (2), Crocker and Harrington (4) and others whose work is touched on later in this review, shewed that catalase activity paralleled respiratory activity. This fact suggested to some that catalase activity might be used as a measure of viability in seeds.

Nemec and Duchon (21) published some work based on this principle, but gave rather scanty particulars as to their method. They determined the volume of O_2 split off from 15cc of 3% H_2O_2 by 2 gms. of ground farinaceous and 1 gm of oleaginaceous seeds in a given time. By running a control in duplicate, using flour of the seed in

question which had been heated to 100° C for 20 minutes on the water-bath, and subtracting the volume of O_2 liberated by the control from that liberated by the experimental sample, they obtained a figure expressing the catalase activity of the seed which they found to be directly correlated with the germination. They then prepared a graph from which the viability might be read, the catalase activity as obtained by this method having been determined.

In a later paper (22) these authors traced again the correlation of loss of vitality with loss of catalase activity, which, they concluded, represents a vital indicator enabling one to see in only a few minutes whether the seed examined has a high germination or not. They worked entirely with seeds of low vitality through age, using barley, wheat, oats, peas &c. Extensive figures of numerous experiments are given, and graphs where the percentage of germination is plotted against O_2 liberated in 5 minutes, are shewn. Several of the tables of figures include data shewing the progress of the reaction at regular intervals and from these data, using the following modification of the mono-molecular formula ($K = \frac{1}{\sqrt{t}} \log \frac{a}{a - x}$) in which $\sqrt{t}$ is substituted for t, they calculated a series of values for K which were very tolerably constant except for the first few readings of any one determination, and which they used as an expression of the catalase activity of the seed instead of the volume of O_2 liberated. In the above formula, K should be constant with varying values of t and x, where t is the time in seconds at which the reading was taken, x is the volume of O_2 liberated in that time and a is the initial concentration of the O_2 in the H_2O_2 capable of being liberated. This constant K they found to be correlated with the percentage of germination, and prepared graphs from which the latter might be read, the former having been determined experimentally.

It will be shewn later that some other investigators have failed to find this direct correlation claimed by Nemec and Duchon; but here, a review of the literature on the functions of catalase and the determination of its activity will serve as an introduction to papers dealing with catalase activity and seed viability.

Appleman (1) working with potato catalase found that rapid deterioration of its activity set in after making

the preparation, unless certain precautions were taken. These were to grind the material with $CaCO_3$, promptly to dilute the extract in the proportion of 1:10, and to keep it at 20°C, or less. He pointed out the necessity of grinding for a uniform time in order that the different preparations might be comparable, and used, as the basis of his comparison, the volume of oxygen liberated after a given period. He confirmed the existence of the insoluble (α-catalase) and soluble (β-catalase) forms mentioned by Loew (15, quoted from Appleman), based on the passage of approximately 50% of the catalase through a filter paper, though none passes through a Pasteur-Chamberland filter. He found that potato catalase is very sensitive to temperature, being completely destroyed at 50°C as compared with 65-80°C for other forms, the destruction commencing at temperatures over 20°C. As the result of his experiments he concluded that catalase can only decompose a limited quantity of peroxide, and that catalase activity appears to bear some relation to respiration.

In a later paper previously quoted (2) this author concluded that while oxidase activity does not, catalase activity does shew a very striking correlation with the respiratory activity of the tubers.

Crocker and Harrington (4) describe two types of dormancy in seeds. In the one there are certain fundamental changes necessary before growth can start, which are favoured by low temperature (5°C) and abundance of oxygen and moisture. The other is of the hard seed-coat type. They also describe a secondary dormancy imposed by the structures enclosing the embryo, as for example, in Johnson grass (Sorghum Halapense (L.) Pers) which fails to germinate when kept moist at constant temperature, but which requires dry storage for after-ripening.

In making their catalase determinations they used a given weight of the finely ground seeds sifted through bolting cloth. The strength of the H_2O_2 was determined by using an excess of catalase material which they found acted as a buffer to counteract the adverse effect of the acidity of the peroxide, and released the O_2 quantitatively. They used 1.5 gm of material in the case of crimson clover seed in 5cc of H_2O, and 2cc H_2O_2 diluted to 5cc. They point out the necessity for neutralizing the acidity of the peroxide, discussing the effect of varying degrees of acidity on the catalase activity of Johnson grass, and conclude that an excess of $CaCO_3$ is as effective as exact neutralization to phenol-phthalein with NaOH. This point is further elaborated

with other seeds. In considering the effect of the degree of pulverization they found that excessive agitation caused a partial destruction of the catalase and that the degeneration of the enzyme was relatively quick after grinding if stored in a dessicator over CaO.

In comparing the catalase activity of different parts of the caryopses of grasses, they found that the embryo shewed much higher activity than the endosperm.

Among the numerous conclusions and points of interest mentioned, the following bear directly on the scope of this thesis.

Catalase activity paralleled respiratory activity in seeds much more closely than viability or vigour. For equal weight immature grains shewed much higher activity than mature, this preponderance being maintained even after drying and storage, but the same number of immature and mature seeds of Johnson grass (separated by means of the blower) shewed approximately equal catalase activity.

The drying of seeds was shewn to lower catalase activity, which was also observed to become less as seeds aged. This however was concluded to be not true of all seeds, as it was not observed in the case of Amaranthus retroflexus. They failed to find that catalase activity was directly correlated with vitality. Two graphs were prepared, one shewing two curves illustrating the relation between germination and age, and catalase activity and age respectively; the other shewing two curves illustrating the relation between germination and the number of hours heated at 81°C, and catalase activity and number of hours heated at 81°C respectively. Comparison of these two graphs in the opinion of the authors, "strengthens the coagulation conception of age degeneration".

Seeds that after-ripen with dry storage but without self-imposed dormant embryos, either appeared to shew no change in catalase activity (e.g. Amaranthus spp.) or a lowering thereof (as in the case of grasses) with after-ripening; while those that after-ripen in the germinator at low temperature, having self-imposed dormant embryos, seemed to shew increased catalase activity with after-ripening.

Seeds which had been treated with heat to lower their vitality shewed a greater fall of catalase activity than of germination in the shorter periods of treatment, but

the reverse in the longer periods in the case of Johnson grass. In air-dried seeds of Amaranthus spp., the catalase appeared to be comparatively heat- and time-stable, the substances connected with viability, comparatively heat- and time-labile, while with Johnson grass the reverse was the case. This latter shewed a loss in catalase activity under storage in a germinator at 20°C, but the loss was less marked at 7°C. The longevity of dormant seeds appeared to be limited by the exhaustion of stored foods by respiration, the intensity of which is much reduced during dormancy along with catalase activity. With the germination of the seed, the catalase activity increased. The proportion of the soluble β-catalase varied with different species, being responsible for about 50% of the total activity in Amaranthus retroflexus, and about 14-30% (depending on the filter used) in Johnson grass.

In a paper designed to shew the inadequacy of the then existing methods of measurement of catalase activity, and to propose a new method, Becking and Hampton (3) criticised a number of methods in use at that time or proposed.

They point out that the measurement of the height of the foam produced during the reaction (method of Palladin) is rendered useless on account of the fact that this height varies with the purity of the enzyme. The titration method with $KMnO_4$ fails to shew clearly either the end point or the beginning of the reaction in many cases, the beginning being considered as being at the conclusion of the latency period.

Discussing the manometric methods, they note the danger of over saturation of the substrate with gas. This and the fact that the solution contained a great part of the oxygen was overcome by shaking and by the use of a shallow layer of fluid with a large surface, since shaking does not injure the catalase in the short time of the catalase reaction, even though when prolonged it does. The fact that the pressure becomes higher during the reaction, another objection which had been made to manometric methods, was disregarded as they found the effect to be negligible.

Another important criticism of nearly all methods of catalase determination was advanced. Recalling the definition of an enzyme as a substance that changes the velocity of a reaction, the only method theoretically justifiable would be to determine the time in which the reaction is com-

pleted under the influence of the enzyme.

The authors developed an autographic method which provided a complete record of the reaction and gave a satisfactory means of measuring the reaction-time. The reaction vessel (providing a large surface to the thin layer of the substrate) was connected to a mercury manometer by a ground glass joint. In the free arm of the manometer was a wooden float bearing a convenient support for a glass pen. This recorded on a cylinder in the usual way, the speed of the cylinder being 1mm. in 1 3/8 seconds at the periphery.

Discussion follows as to the chemical nature of the decomposition of H_2O_2; they consider that there is evidence of two successive reactions and that the α - and β -catalase of Loew are two successive stages of peptisation of the same substance.

In considering the comparison of the action of two different quantities of enzyme, since they found that there was destruction of catalase during the reaction, they point out that the reaction velocity was not constant; hence they conclude reaction-velocity is a misleading test for the strength of an enzyme. On the other hand, the amount of peroxide decomposed gives different ratios after different lapses of time. These considerations confirm their previous conclusion that the times required to complete the reaction should be directly compared.

Using their autographic method they found that the reaction time was inversely proportional to the amount of enzyme and, with a constant quantity of catalase, directly proportional to the quantity of peroxide.

They worked out the reaction velocities of a number of different experiments and found that the expression:-

$$\frac{\text{Reaction velocity} \times \text{number of cc. peroxide}}{\text{Number of cc. enzyme}}$$

was fairly constant, but point out that it would require 39 determinations of this sort to prove what one determination of the reaction time gave, viz. that the reaction follows the Law of Mass Action.

They tried further to compare the curves obtained with mathematically constructed logarithmic curves, using the

equation:-

$$t = C \log \frac{a}{a - x}$$

and found:-

Calculated from curve.	Amount extract.	C X E.
0.15	4 cc.	0.6
0.20	3 "	0.6
0.30	2 "	0.6
0.60	1 "	0.6
1.20	½ "	0.6

and concluded that this method would probably be the most practical and accurate.

They found that the latency time X amount of enzyme seemed to be more or less constant but felt it was premature to draw any conclusions from this.

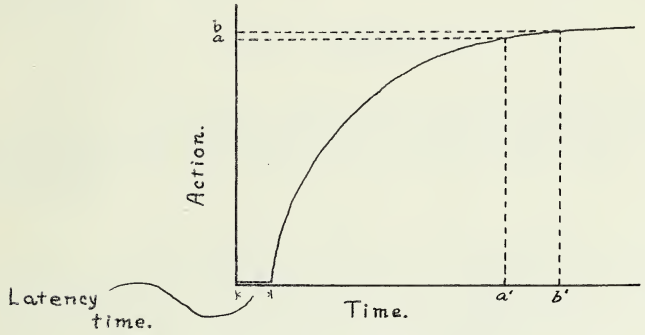


Fig. 1.

Fig. 1 shews the type of curve obtained on an autogram. It occurred to the present writer that it must be a matter of considerable difficulty to determine when the end point in time had been reached, though there is less chance of error in reading the "action" end point (cf. a & b with a'& b') The authors calculated the volume of oxygen actually liberated by subtracting the known volume of the container less that of the extract and H_2O_2, from the observed final volume (allowing for the pressure set up by the column of mercury) and comparing it with the known volume of oxygen the peroxide was capable of liberating. Having determined the point on the autogram representing maximum oxygen liberation, the corresponding time was taken as the reading.

In a study of the depressive effect of successive

doses of peroxide on a given quantity of extract they found that the action of the catalase did not vary with its dilution nor with the quantity of the peroxide, but only with the absolute quantity of the enzyme itself; and that in the particular experiment under consideration the peroxide destroyed ± 10% of the catalase during each successive reaction.

In a very thorough study of the catalase reaction Morgulis (16) used a crude liver catalase preparation, but found that the data obtained applied to catalase from many sources. The peroxide was standardized by titration with $KMnO_4$ and its pH adjusted to 6.9. The author points out the error introduced by the use of peroxide with an acid reaction. He found the optimum pH for the catalase reaction to be 7.0 but at this pH the H_2O_2 decomposes somewhat, spontaneously, while at 6.9 the catalase shews almost the same activity as at 7.0. In experiments to determine this point the quantities of catalase and peroxide were so adjusted that the curve of oxygen evolution followed the isotherm of a bi-molecular reaction,

$$K = \frac{1}{t} \cdot \frac{x}{a(a - x)} .$$

In this first series of experiments the reaction was found to run true to the course of a bi-molecular curve at pH values of from 6.4 to 8.3, but not at acidities exceeding 6.4.

Increasing the concentration of the H_2O_2, the volume being constant, with a given quantity of extract had the effect of increasing the volume of oxygen liberated from gram-molecular concentrations of 0.16 to 0.32, though the percentage of peroxide decomposed was less with increase of concentration. Gram-molecular concentrations in excess of 0.32 had the effect of diminishing not only the proportion of peroxide decomposed but also the actual amount of oxygen liberated. In addition to these effects, the reaction proceeds more slowly with increase of concentration of H_2O_2. From the curve illustrating these data it may be assumed that decomposition would be complete with a concentration of 0.14 M. H_2O_2.

Discussing the depressive effect of an excess of peroxide on the catalase reaction he concludes that catalase is not destroyed through oxidation by the peroxide since even a very large excess of the latter does not prevent the reaction. Nor does he find this depressive effect to be due to some incidental impurity which becomes present in sufficient quantity to retard the reaction when excess of peroxide is employed. The actual reduction in the volume of the oxygen liberated when the concentration of H_2O_2 exceeds a certain amount leads to the conclusion that the catalase reaction is a reversible one.

The maximum oxygen evolution was found to occur when the relative amounts of catalase and peroxide were so adjusted that 65-70% of the latter was decomposed.

Using a moderate excess of peroxide he found that the reaction came to a stop after partial decomposition. With the addition of a further dose of catalase the reaction went to completion. Then, using a constant quantity of catalase and adding the peroxide in two doses, the first dose was completely decomposed, the second partially and the proportions having been adjusted to conform with the previous experiment, it was found that the total volume of oxygen was the same as before. Another variation of the same experiment was tried in which the same quantity of peroxide was used as in the first, but the first dose of catalase was added in two portions. These two stages made no difference to the total volume of oxygen produced.

He concluded that the catalase is used up in the reaction and that it reacts with a definite quantity of the peroxide.

In a further series of experiments he found that there was a direct proportionality between the amount of catalase and the amount of oxygen it produced from a given quantity of peroxide. This was true only for absolute amounts, changes of concentration alone having no effect.

In a curve illustrating the effect of an increase in the quantity of peroxide with a constant quantity of catalase, he found that the curve of oxygen evolution had a smaller slope for every new increase in the amount of H_2O_2 employed, which is significant since if the diminution were due to an oxidation of the catalase, a proportionately greater destruction of it would be expected with the greater amount of peroxide.

The reaction velocity was found to diminish in the early stages with increase of concentration of H_2O_2, using the reciprocal of the time as a measure of the rate. In the later stages the rate became equalized except with a concentration of over 0.36 gm Mol. when the retardation was very marked throughout.

When the absolute amount of peroxide and the absolute volume of reacting liquids were unchanged but the relative concentration of the catalase was increased, the rate remained practically constant; but when the amount of the catalase was increased, the relative concentration remaining unchanged and the absolute amount of the peroxide being the same, the rate varied directly with the amount of the catalase. He concluded that the reaction rate depends directly on the quantity of catalase used, while the effect of the peroxide is to limit the rate.

In discussing the chemical nature of the catalase reaction he states that the course of the reaction seems to depend solely on the quantitative relation between the available catalase and the peroxide. He finds three stages. When the conditions are such that the decomposition falls between 95 - 100% of the peroxide, the reaction follows a mono-molecular course and the formula $K = \frac{1}{t} \log \frac{a}{a - x}$ applies; when the decomposition falls between 68 - 82% the reaction is bi-molecular, the formula $K = \frac{1}{t} \cdot \frac{x}{a(a-x)}$ applying; while a more or less intermediate zone exists between 88 - 93% decomposition, when the reaction is one and one half molecular, and the formula $K = \frac{1}{t} \cdot \frac{\sqrt{a} - \sqrt{a-x}}{\sqrt{a} \times (a-x)}$ applies.

It was necessary to change the quantities very little to produce appreciable differences in the value of K. Three graphs are shewn in which the experimental curves are plotted with the theoretical curves calculated from the average value of K for each respective experiment and shew a striking similarity between the two.

The use of very large quantities of peroxide is criticised on account of the depressive effect unless very large quantities of catalase are employed. On the other hand, when comparison is made of the catalase activity of preparations of presumably different strengths, the depressive effect is much greater in the case of the weaker sample and differences will be exaggerated.

He concludes that the entire method of comparing several samples of catalase on the basis of the amounts of oxygen liberated is of questionable accuracy, and recommends that the comparison should instead be made between the respective quantities of catalase preparation required to set free

the same amount of O_2 from a given quantity of H_2O_2, and that further, the reaction be adjusted to follow some definite course approximating 75% peroxide decomposition.

Repeating the experiments of Nemec & Duchon, with different varieties of pea, De Vilmorin and Cazaubon (26) found a distinct correlation between the diminution of their catalase activity and of their germination on account of age. In the case of the seeds of certain trees however, notably pine and larch, in which they felt this reaction would be of particular value because of the slow germination of these seeds, they found that there was still considerable catalase activity even in dead seeds, while it was much reduced when the seeds were heated at 100°C for one half hour. Hence they concluded that the method was inapplicable to these species.

Heinicke (10) working with apple-leaf tissue pointed out the importance of variations in concentration of the substrate, particularly in the case of catalase material. He found that since, by increasing the quantity of H_2O_2 used, the volume of O_2 liberated was increased somewhat, but not markedly, it was important to have an excess of peroxide.

Agitation of the preparation, a liquid suspension, did not degenerate the catalase of apple-leaf tissue. In making the determination, the rate of agitation varying from 30-90 shakes per minute, did not affect the speed of oxygen liberation.

On letting the preparation stand, the catalase activity increased up to one hour, after which the increase became so slight that it might be disregarded, and retained its full activity up to four days. Without preliminary neutralization with $CaCO_3$, however, the degradation was very rapid.

Shull and Davis (25) working with seeds of Xanthium found that catalase activity was not strictly proportional to the amount of material used. With this species, they found no variation in activity as a result of dry storage, but that germinator storage at a temperature too low for germination increased respiratory and catalase activity. Seeds collected from the field at weekly intervals shewed a progressive decline in catalase activity till about the middle of April, when it began to increase as the natural germinating season approached.

Heinicke (11) in a paper later than the one previously quoted, in discussing the preparation of material

pointed out the advisability of trituration with water, the time of grinding making no difference provided it was done wet; the probable cause of the lowering of catalase activity with excessive grinding in Crocker and Harrington's experiment with Johnson grass being that it was done dry.

He tested his peroxide with MnO_2 using as the index of activity the time required to liberate 5cc of oxygen. He found a retardation of the reaction if the 5cc of oxygen liberated represented more than 1/5 or 1/6 of the available O_2 in the peroxide. On the other hand the larger the amount or the greater the strength of the peroxide, the slower the initial rate of catalase activity, but the more rapid the subsequent liberation of given quantities of oxygen. In such cases he found that the volume of gas in the burette might either be reduced by as much as 0.5cc when the liquids were first mixed, or remain the same for the first 5-15 seconds in spite of continual shaking. This "latency time" while frequently observed, does not always occur.

After the first dose of peroxide was completely decomposed, he found that a second was split more slowly, whether by catalase or MnO_2. The effect of the dilution of the liquids in the reaction chamber was to retard the liberation of the O_2 from the H_2O_2, both by his apple bark preparation and by MnO_2, such dilution of a weak preparation resulting in a more than proportional loss in catalase activity, while doubling the quantity of the preparation reduced by more than one half the time required to liberate a given quantity of oxygen. The stronger the original preparation, whether of catalase or MnO_2, the more nearly proportional was the activity of diluted or increased quantities.

Ota (23) concluded that respiratory and catalase activities run parallel throughout the dormancy period, in seeds of Xanthium.

Knott (13) working with leaf tissue, principally that of spinach and tomato, used the following technique. One gram of discs of one centimeter diameter cut from the leaves, was ground with an equal weight of $CaCO_3$ in a mortar with 2cc of water and a pinch of pure quartz sand for two minutes. The paste was washed into a bottle with 18cc H_2O (or presumably made up to 20cc total). After thorough mixing, 2cc were immediately withdrawn and placed in one arm of the dry reaction tube, in the other arm being placed 2cc H_2O_2 (equivalent to 26cc available O_2) previously neutralized with a slight excess of $CaCO_3$. During the course of the reaction the levelling bulb was moved to keep the reaction under constant atmospheric pressure. His rate of shaking was 240

complete excursions per minute, but he found no variation in the total rate of liberation of oxygen when the speed was varied from 220-300, though the initial rate was faster with the higher shaking speed.

A constant temperature bath operated at 20°C was used, the reaction vessel being immersed therein for three minutes prior to shaking. Check tests were made to within an accuracy of 1%.

He allowed the reaction to proceed beyond the 5cc used by Heinicke, since he found that catalase activity in two tests might run parallel up to this point but diverge markedly thereafter.

In discussing the amount of H_2O_2 to use, he stated that it might in certain cases be a limiting factor when the oxygen liberated was more than one fifth of the total available, but he had not found it so till 60% of the O_2 was released. By increasing the quantity of H_2O_2, the stronger catalase preparations shewed a slight increase in liberation of oxygen, less strong preparations being unaffected, while weak preparations shewed a retardation.

The problem often arose as to how a catalase preparation might be kept with its activity unimpaired. Held at room temperature, it decreased in activity up to a point when it started to increase, after about 50 hours. This secondary activity was shewn to be due to bacteria. He tried the use of 1% toluene, which checked bacterial growth but also affected the rate of the catalase reaction. The problem was finally solved by employing low temperature storage, and he found that placing the bottles on ice immediately after trituration and dilution appeared to be the best method of keeping over preparations of celery and spinach, but that tomato catalase lost its activity slightly during 24 hours, even when on ice. One other factor was found to have an influence on this point. The exposure of the preparation to air after cold storage removed some inhibiting factor and thereafter the catalase reaction was found to have been accelerated. The weaker the catalase reaction the greater was this effect, which reached its maximum after 6 - 8 minutes exposure to air. This effect was thought to be due to the greater solubility of CO_2 at the low temperature.

More recent studies in the determination of seed viability or age by methods other than the germination of the seed, or the catalase reaction, include those of Fick and Hibbard (8) and Munerati (20). The former based their method on the measurement of the conductivity of water

in which seeds had been soaked. The greater diffusion of the salts from seeds of low viability than from those of high, brought about the greater conductivity of the soak water. Using varying mixtures of old seed of 3% germination (since artificially killed seed were found to be unsuitable) with fresh seed having 92% germination, some very fairly consistent results were obtained. Timothy and clover was the material used. Munerati found that the optimum temperature for germination was correlated with the age of the seed.

Davis (6) failed to find a close correlation between catalase activity and germination as reported by Nemec and Duchon. Since, however, the loss of catalase activity seemed to lag behind the loss of viability, it occurred to him that its disorganization might be brought about in some manner that would not affect the catalase of viable seeds. He found that soaking in warm water effected this. Using the ratio:- cc. O_2 liberated by soaked: unsoaked, he found that this ratio was more or less equal to unity when the germination was high, but less when the viability was low.

Instead of a given weight of material, this author used a given number of seeds. He found that in choosing between a short soak at relatively high temperature and a longer soak at a lower temperature, the former was preferable since disorganization of the catalase was more complete and there was less chance of its increase with increase of respiratory activity. The catalase content of viable lettuce seed was not reduced by soaking for one hour at 54°C, (high temperature, short soak). Some seeds responded much more completely to the high temperature soak than to the low. He used as his basis of measurement the volume of O_2 liberated after ten minutes.

With seeds shewing high catalase activity he recommended grinding a large number with repeated additions of 20cc. or more of water, and working the mass through a 60 mesh sieve till 250cc. had been obtained. After thorough agitation a 5cc. aliquot to which 5cc. of water were added, was taken for each run.

He found that the seed axis and especially the hypocotyl portion of the embryo appeared to be the first to succumb to devitalizing factors. In cases where this was true and the cotyledons or endosperm formed the bulk of the seed, the difference in catalase content of the dry and soaked seeds might not be sufficient to indicate the true condition of the viability of the seed. In such cases it was recommended that the catalase be determined on the epicotyl and hypocotyl without storage parts.

Where the seed was badly infested with fungi the test was found to be of no value, as the living fungi bear part of the catalase and protect it against decomposition in the high temperature soak.

The advantages claimed for the method are, that it is unnecessary to know the catalase content of the same kind of seed of high viability; the quick estimation of the viability of dormant seeds is possible; viability of quickly germinating seeds can be still more quickly estimated where special dispatch is necessary; and for checking up erratic results obtained by germination methods.

In a study of the catalase relations of rice under different germination conditions, Morinaga (19) found that there was only about one tenth as much catalase in dry seeds of rice as in those of wheat, oats, barley and rye. Rice germinating anaerobically shewed no increase in catalase, but a gradual increase was observed in the course of germination in a medium with a reduced supply of oxygen, while under aerobic conditions of germination the catalase activity was high, being about seven-tenths as much as that in the germinating grains of wheat, barley and oats. Hence it was concluded that the ratio of increase of catalase activity is a function of the free oxygen in the medium.

The free oxygen was found to affect the development of the radicle and plumule and also of the chlorophyll of the latter. The catalase once having been increased under aerobic germination conditions, decreased when the plant was returned to anaerobic conditions, though the growth continued. He found further that where the catalase had been increased by germination under aerobic conditions, much more oxygen was used by the seedlings than in the reverse case, but the carbon dioxide relations were unchanged.

In a general discussion as to the value of catalase as an indicator of seed viability, Gračanin (9) points out that contradictory results have been obtained by different authors. He found with certain seeds a diminution of catalase activity with loss of vitality when such loss of vitality is occasioned by age. In plant tissue which has lost its vitality however, he distinguishes, after Grafe, between "dead" and "disorganized" tissue. In the former case while vitality is gone, the enzymes still retain their properties to a certain extent, while in the latter the enzymes are lost also. In order to bring about these two conditions in seeds, he soaked them in various solutions. Thus 0.05 M NaCl and 0.001 M $ZnSO_4$ killed the cells (leaf tissue of Mnium undulatum) without disorganizing the catalase; 0.001 M $CuSO_4$ killed the cell and reduced

catalase activity; while 0.001 M $FeCl_3$ and 0.001 M I destroyed both life and catalase activity. Similar results were obtained with seeds of four species.

He concludes that while loss of catalase activity indicates loss of vitality in seeds, the converse does not hold necessarily, and points out how erroneous conclusions could be reached by judging the vitality of seed by the catalase test, if such seed had been shipped by ocean and become injured by the salt water.

Faced with the problem of determining the relation between the vigour of seeds and the condition of the distilled water in which they had been soaked, Hottes and Huelson (12) carried out a large number of experiments using the Abbé refractometer and the Leitz nephelometer. The method used was to soak 5 or 10 grams of the seed in 50 cc. of water in well stoppered bottles for 48 - 72 hours at 30°C. Two or three drops were used for the reading. There was found to be an inverse correlation between the refractive index and germination as also between the colloidal index and germination, but the latter correlation was better. As a standard for the colloidal index readings 0.5% c.p. soluble starch was used dissolved in 0.5% sodium toluene para-sulphochloramide.

They found that the coefficients of correlation tended to increase inversely as the percentage germination -- an advantage, since it is usually the low germinating samples which are in doubt. The authors think the method may prove useful in determining the viability of grains and seeds having a fairly large endosperm.

The two following papers have been left for discussion at this point on account of certain mathematical features they present, which it was felt made it advisible to discuss them together, though the discussion of the more general features will be considered at the same time.

Morgulis and Beber (17) working with a beef kidney preparation, shewed in a previous paper (16) that the optimum temperature for catalase activity lay between 0°C and 10°C. It is to be noted that in the work of these authors, the criterion as to catalase activity is not the rate of liberation of oxygen as with most of those previously quoted, but the total amount of oxygen that a given quantity of the preparation is capable of liberating. Hence, although the reaction proceeds much more slowly at the low temperatures mentioned, it does so much more completely, the difference being due to the partial destruction of the catalase at the

higher temperatures.

The material used for the experiments dealt with in this paper was a semi-pure beef kidney preparation which, having been maintained at pH 7.0 by the use of the Kolthoff phosphate-borax buffer, had retained its full strength for three years. The experimental temperatures ranged from 0°C to 30°C. The method of temperature control was an improvement over that previously used and was very accurate. As the experimental temperature was increased it was found necessary to increase the catalase concentration to make up for the destruction of the catalase during the experiment.

Fig. 2., roughly copied from the paper under discussion shews the relation between catalase activity and temperature with varying amounts of the catalase preparation.

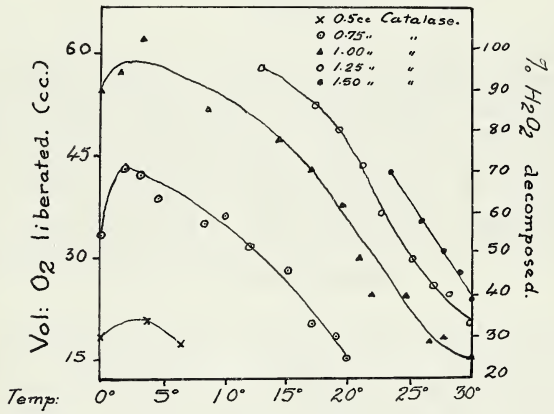


Fig. 2.

The optimum temperature is found to lie between 1°C and 3°C. The variation between these temperatures is so slight that the authors considered that the mean, 2°C, might be set as the optimum temperature.

In Fig. 3., also copied from their paper, the results of the same series of experiments are arranged in such a way that the percentages of H_2O_2 decomposed are plotted against the relative catalase concentrations. The straight line graphs so produced are therefore catalase isotherms.

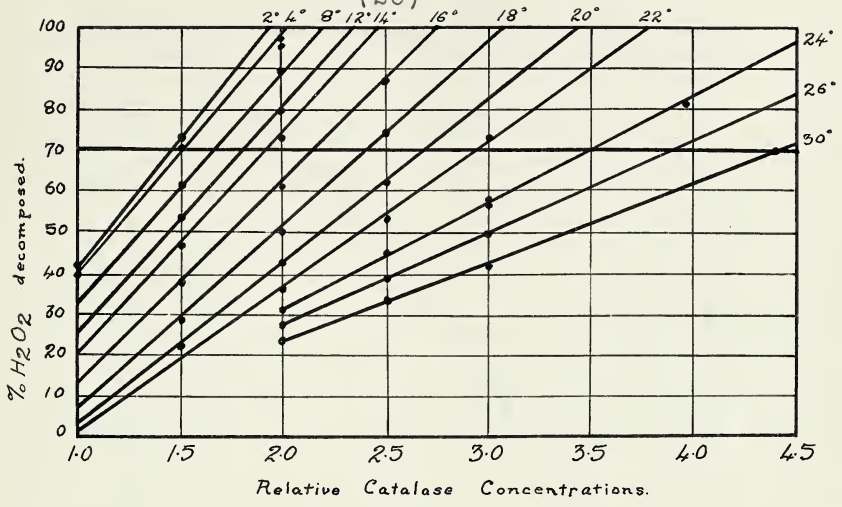


Fig. 3.

In the paper previously quoted (16) Morgulis found that the course of the catalase reaction at the point where 70% of the H_2O_2 was decomposed, followed exactly that of a bi-molecular isotherm, and had previously been proposed as a means of standardizing catalase preparations. By drawing the horizontal line corresponding to this degree of peroxide decomposition, the abscissa shews the relative catalase concentration which will manifest this activity at varying experimental temperatures.

Assuming that all of the catalase is active at the optimum temperature (2°C.) it is possible to calculate how much is destroyed at a higher temperature; for, regarding the amount of catalase which at 2°C effects 70% decomposition as one enzyme unit, the relative amount which is still active at any other temperature may be obtained. If the values of the relative catalase activity thus calculated are plotted against their corresponding temperatures, the curve, Fig. 4 (also copied) is obtained, which corresponds to the equation:- $y = 0.58+0.425 \sin 90/19 (10 - t)$, when y equals the relative catalase activity and t the experimental temperature.

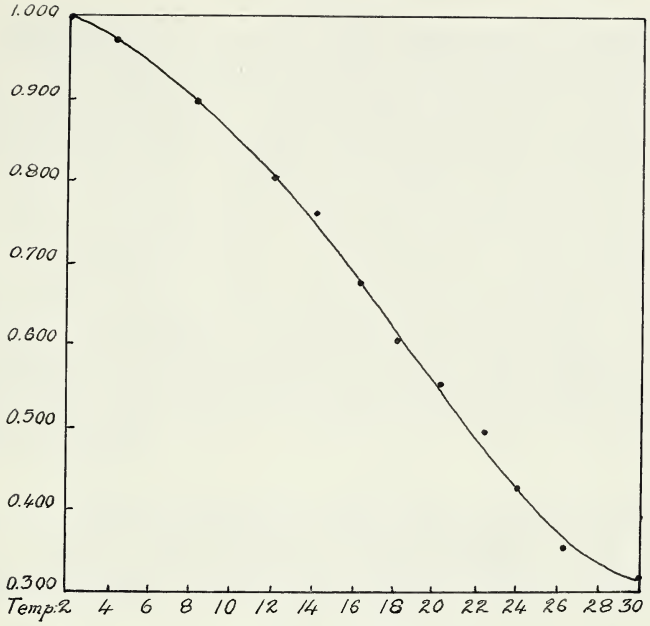


Fig. 4.

This expression holds good up to a temperature of 24°C, but beyond this point there is a divergence. While this divergence is of no practical value, since higher temperatures than this are never employed in catalase determinations, there is a theoretical interest in this deviation. In a previous paper (18 quoted from 17) it was shewn that catalase destruction is a mono-molecular reaction with a temperature coefficient which increases moderately for higher temperature ranges; the temperature coefficient for the catalase reaction however having no fixed value, but increasing greatly as the experimental temperature rises. It follows from the new observations that 24°C is a critical temperature for the catalase reaction, the rate of the catalytic reaction being so much greater than that of the enzyme destruction reaction that the volume of oxygen set free becomes progressively greater than the theoretical amount for temperatures above 24°C.

This curve makes it possible now to find the actual number of enzyme units no matter at what temperature the experiment is performed. Experimenting at temperatures as low as 2°C is not practicable and even if it were it would not be desirable, for the reaction at this temperature is very

slow, whereas at 16 - 20°C the reaction is complete after from 30 to 90 minutes. The results obtained at any of these temperatures may easily be corrected for the catalase destroyed at the temperature used. It is necessary to determine the percent H_2O_2 destroyed by at least two different catalase concentrations (which should produce between 50 and 80% decomposition but in any case not more than 90%), whereby the catalase isotherm may be established and the catalase concentration producing 70% decomposition determined. The relative activity at the experimental temperature may be read from curve Fig. 4. The quantity of catalase producing 70% decomposition (Q) divided by the factor for relative activity (A) at the temperature of the experiment gives the actual amount of the catalase used in the test; or if Q/A at 2°C, where A = 1, is considered as the enzyme unit, the figure obtained is the number of enzyme units.

They concluded that any catalase preparation could thus be standardized on a strictly quantitative basis, which is easily and exactly reproducible, and in experiments designed to prove this point, found their conclusions justified.

In a paper dealing with the relationship between catalase activity and respiration of germinating seeds Rhine (24) after discussing the properties and probable functions of catalase and various theories concerning the same, pointed out that the fact that in many cases a parallelism had been found between catalase activity and respiration, has lead the former to be determined as a measure of the oxidation rate; but that there were discrepancies which it was the intention of her paper to solve.

The reaction vessel was operated in a water-bath maintained at 21 - 22°C, and all results were calculated to standard temperature and pressure. In all experiments 5 cc. of Oakland dioxygen neutralized with N/10 NaOH was the quantity used. The tissue was ground with $CaCO_3$ and sand and used quantitatively with 15 cc. H_2O. When the temperature of the bath was reached the peroxide was added, and shaking commenced 15 seconds thereafter. One 10 minute reading was taken, excess pressure being avoided by adjusting the level in the burette. The degree of pulverization, which was done dry, was determined according to the operator's "best judgment", being as nearly as possible equivalent to grinding dry wheat for four minutes.

There follows a discussion of the objection of Morgulis et al " that a time reading, arbitrarily chosen for the measurement of the catalase-hydrogen peroxide reaction, cannot afford an accurate measurement of catalase activity. Under the law of mass action, an arbitrary time limit would

not express the results of comparable stages in the reaction when the amount of one or more substances was varied. Thus when the proportion of catalase to H_2O_2 is high, a 10-minute reading would express the results of a much greater part of the entire reaction than it would when the amount of catalase is relatively small". The authoress shews a table in which this is illustrated. She goes on to point out that Morgulis stated that a great excess of either of the reacting substances exerts a depressive influence on the reaction, as a result of which he argued that catalase determinations could only be compared by comparing the amounts of catalase preparation required to liberate equal volumes of oxygen. The impracticability of this is pointed out and a remedy suggested. A series of catalase determinations were made involving a wide variation in the amount of the catalase preparation used (in this case wheat), shewing sufficient excess of catalase at one end and of H_2O_2 at the other for this depression to be produced. Having calculated the volume of oxygen liberated per gram of material, the highest value was taken as unity and the necessary correction factors calculated as shewn in the following table (quoted from Rhine):-

TABLE I.

Weight in grams.	Cc. O_2 liberated in 10 minutes.	Cc. O_2 per gram.	Factor.
0.02	0.49	24.55	1.34
0.04	1.01	25.55	1.30
0.06	1.56	26.00	1.26
0.08	2.06	25.76	1.28
0.1	3.01	30.10	1.07
0.2	6.38	31.94	1.03
0.4	12.92	32.30	1.02
0.6	19.77	32.95	1.00
0.8	24.55	30.69	1.06
1.0	30.80	30.80	1.07
1.2	35.82	29.84	1.13
1.4	39.49	28.20	1.14
1.6	42.02	26.26	1.25
1.8	44.49	24.71	1.33
2.0	47.82	23.91	1.37

All her further results were corrected with these factors. Another table is given shewing similar data but using different volumes of a water extract of rye ovules. These two tables gave curves varying in type, but since in subsequent experiments the ground seed type of material was

used, the former set of factors was employed. All tests were run in triplicate.

She found that while there was a large and immediate rise in respiratory activity when seeds were put under germinating conditions, the catalase activity decreased in the early stages of germination in the case of six kinds of seeds tested; but the initial decrease however was followed by the rise generally reported. Thus the early curve of respiration was found to diverge widely from that of catalase activity.

Catalase was found to decrease slowly, almost to exhaustion by prolonged soaking of seeds in oxygen-free water.

The decrease in germinating wheat was evident both in the embryo and endosperm, the subsequent rise, however, while not confined to the embryo, was much more marked therein.

In ripening seeds the catalase was found to decrease both per unit of wet and of dry weight. It increased per ovule as the seed grew, but after ripening began decreased rapidly with lowering of water content.

The production of catalase was found to be indirectly connected with oxidation, and Loew's suggestion was advanced, that the function of catalase is to remove either hydrogen peroxide or some other substance as yet unknown which the catalase has the power to decompose, and which is formed in the tissues as the result of metabolic processes.

In her opinion catalase activity could only be used as an indicator of metabolism in those cases where there was no rapid change in respiration.

Summary.

A number of biochemical methods of estimating the viability of seeds have been considered. The method involving the determination of catalase activity has been chosen for further study as giving promise of the solution of the problem and also as holding the possibility of throwing light on some of the problems of dormancy and delayed germination.

Literature on the catalase reaction has been reviewed. Among authors who have attempted to use the catalase reaction as a measure of seed viability considerable difference of opinion exists as to its usefulness. Two methods which are claimed by their authors as giving a measure of seed viability have been described. In the one, the difference between the catalase activity shewn by the untreated crushed seed and that shewn by the crushed seed after treatment on the water-bath of 100°C for 20 minutes is taken as the measure; in the other the ratio;- catalase activity of seed soaked at fairly high temperature: that of seed not soaked, is the measure.

The optimum pH for the catalase reaction has been shewn to be 7.0. The method of neutralization of the acidity of the peroxide with excess of $CaCO_3$ and grinding the material with excess of this substance has been used by a number of investigators to produce conditions approaching this optimum and appears to be a satisfactory means to accomplish this end.

A depressive effect due to excess of either the peroxide or the catalase material has been found by several authors.

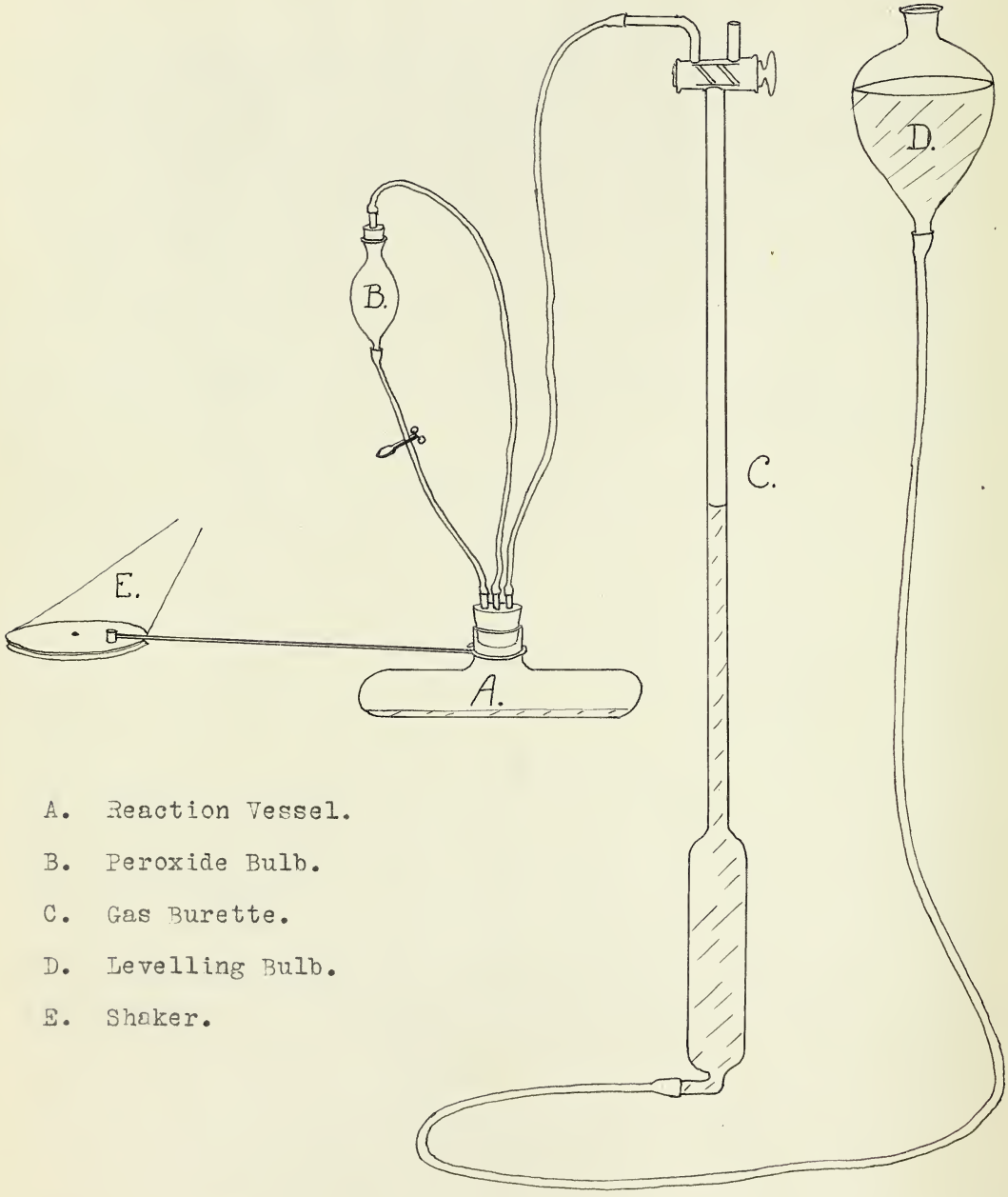
A number of methods have been proposed as a basis for expression of catalase activity. The most practical for the purpose of this work appears to be the volume of oxygen liberated after a given time corrected by a factor to allow for this depressive effect. This is more fully discussed in the next section.

The importance of comparing the activities of catalase preparations under uniform temperature conditions has been stressed. The most suitable temperature from a practical point of view is 20°C though this is not the optimum temperature for the catalase reaction.

Catalase has been reported to exist in a soluble and an insoluble form; while one author considers these two forms to be different degrees of peptization of the same substance.

Respecting the quantity of catalase material to use, some workers have used a given weight, others a given number, of seeds. The latter is satisfactory where the seed is uniform, but the former is perhaps the more generally useful. It is to be noted however in comparing seeds of different degrees of maturity directly, that Crocker and Harrington (4) found that a given number of immature seeds exhibited the same activity as the same number of mature seeds of the same species, though they weighed less.

Apparatus used for Catalase Determinations.



A. Reaction Vessel.

B. Peroxide Bulb.

C. Gas Burette.

D. Levelling Bulb.

E. Shaker.

Fig. 5.

EXPERIMENTAL PART.

Development of a Method for Seed Catalase Determinations.

While some of the methods for the determination of seed viability outlined, which did not make use of the catalase reaction appear to be promising, it was felt that the catalase method not only gave promise of the solution of the problem, but that it might, moreover, throw more light on seed germination problems.

Materials.

It was thought best to confine the following study to one kind of seed in order to avoid undue complications and that the study might be more complete; accordingly spring wheat has been used throughout.

Merck 3% H_2O_2, which in repeated tests was shewn to be very fairly uniform in strength and which could be secured locally, was used.

Merck precipitated $CaCO_3$

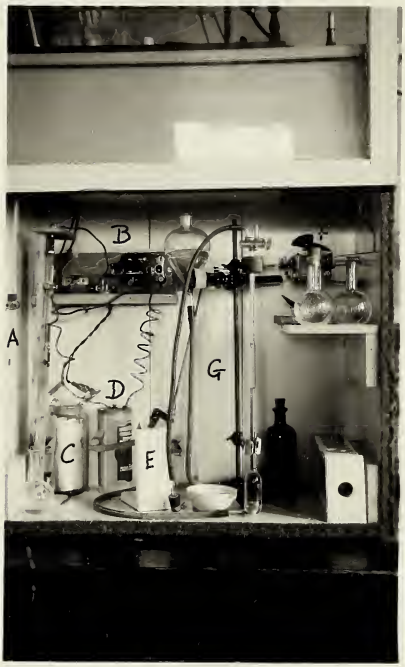
Apparatus.

The apparatus used was similar to that shewn in Fig. 5, the reaction vessel being operated by an electric shaking mechanism, the speed of which could be varied within wide limits.

In the early part of the work an attempt was made to keep the temperature of the laboratory (about 12' by 14') constant at 20° C. by a thermo-regulated auxiliary heater. This not proving very satisfactory the reaction vessel was immersed in a constant temperature water-bath, which was operated by a relay mechanism. This in turn not proving as satisfactory as could be desired, the author constructed a constant temperature cabinet, a cut of which is shewn in Fig. 6, in which the entire apparatus was placed, the same relay mechanism, also home-made, being employed. This effects a temperature control within $\frac{1}{4}$° C. and was set to operate a fraction under 20° C. A suitable worm gear clutch mechanism enables the operator to set the shaker in operation from the outside of the cabinet.

While at first a reaction vessel shaped like that in Fig. 5 was used, this was later replaced by a Y-shaped vessel which was much more convenient.

CONSTANT TEMPERATURE CABINET.



A. Thermostat.
B. Relay.
C. Condenser.
D. Battery operating relay.
E. Rocker.
F. Fan and Gear Box.
G. Burette assembly.

Fig. 6.

Relative Catalase Activity of
"germ" and "brush" ends of Wheat.

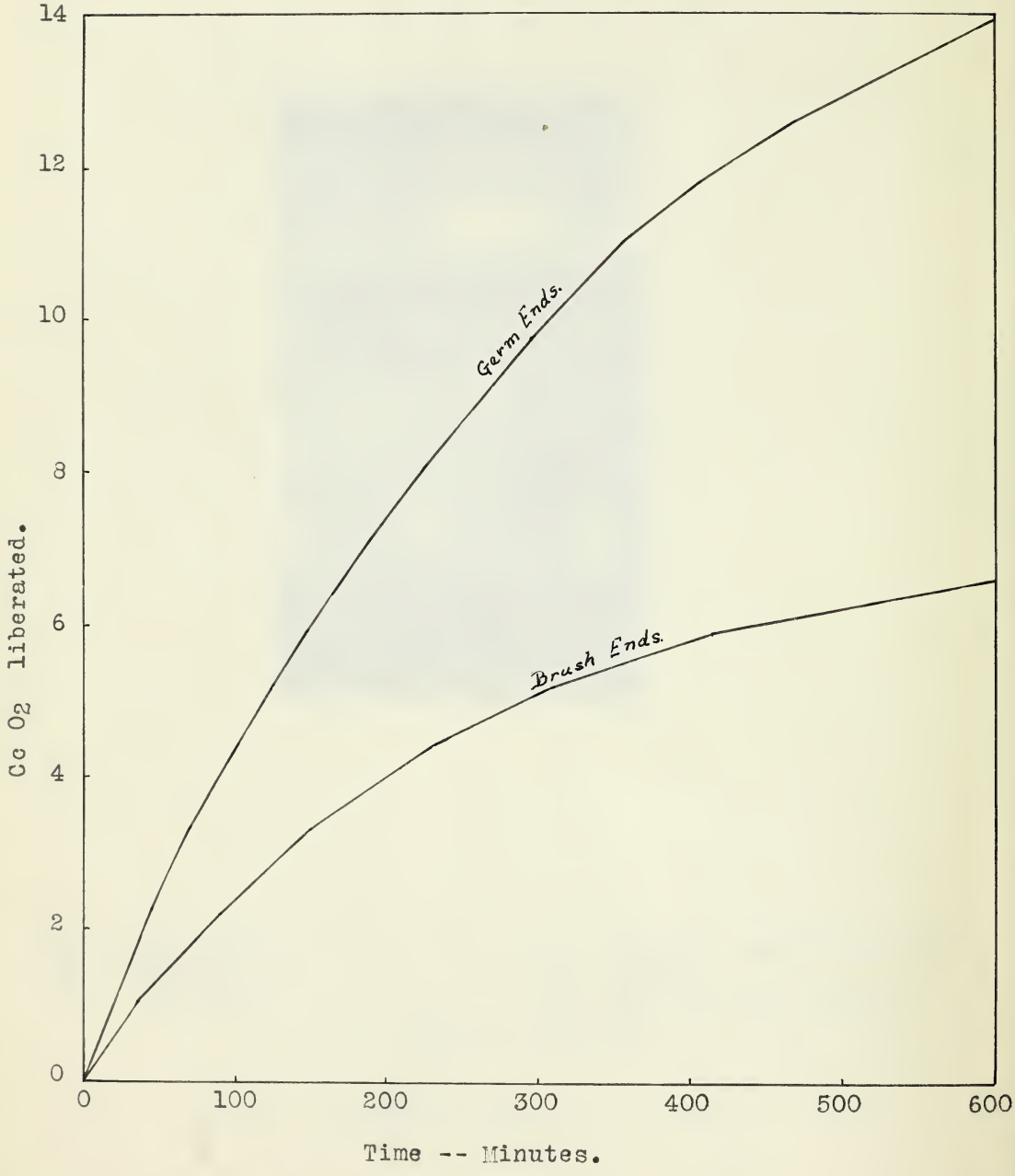


Fig. 7.

During the course of the reaction the leveling bulb was left on its stand, but a graph was prepared from which could be read the actual volume of O_2 displaced corresponding to the volume read on the burette. This correction of course was always made; also, in the latter part of the work all volumes were corrected for barometric pressure.

Experimental.

In regard to the basis for expression of catalase activity, five methods have been reviewed. These are:-

(1) The volume of oxygen liberated after a given time.
(2) The time required to liberate a given volume of oxygen.
(3) A reaction constant derived by the use of a modification of the mono-molecular formula.
(4) The total amount of oxygen a given quantity of catalase is capable of liberating or, alternatively, the amount of catalase preparation capable of liberating a given volume of oxygen.
(5) A modification of (1) above.

While the first of these affords a very convenient measure it has the objection that comparisons are not made between comparable stages of the reaction. Thus strict proportionality between the amount of catalase present and its measurement on this basis cannot be expected. This is illustrated by the curves shewn in Fig. 7. in which the author has compared the activity of equal weights and equal numbers of the germ- and brush-end halves of wheat kernels. After 600 seconds the activity of the brush-ends to that of the germ-ends is as 6.6:14, i. e. the former shews 47% of the activity of the latter; while after 300 seconds the proportion is 5.1:9.7 or 52.6%. Moreover, no regard has been paid to the depressive effect of excess of one or other of the reacting substances.

This latter point is also one affecting the second method. On the other hand since the volume of the oxygen liberated is the same in two comparative readings, the concentration of the substrate is the same and the reactions are hence at comparable stages, yet it is a matter of some moment as to which volume to decide to use as will be made clear from the following considerations. If in Fig. 7. we compare the activities of the

two curves on this basis at 3 cc. and 6 cc. of oxygen liberation, we find that the times required are 132:63 seconds to liberate 3 cc. for the brush- and germ-ends respectively, and 452:152 for the same to liberate 6 cc. Since the activity is the reciprocal of the time we find that at 3 cc. the brush-ends shew 47.7% of the germ-end activity, and at 6 cc. 33.6%. It would be necessary therefore, working with catalase preparations of known strength, to determine the best volume of oxygen liberated to use for comparison. A further objection is that the possible error is much greater in the time reading than in the volume reading as will be seen on examination of the brush-end curve Fig. 7. This is the more marked when the time approaches that required for the completion of the reaction.

The third method mentioned above was the use of the formula

$$K = \frac{1}{\sqrt{t}} \log \frac{a}{a - x}$$

Morgulis (16) shewed that the mono-molecular formula

$$K = \frac{1}{t} \log \frac{a}{a - x}$$

is only applicable to the catalase reaction when the reacting substances are present in such proportion that 95-100% decomposition of the peroxide occurs. As the proportions are so adjusted that smaller percentages of the peroxide are decomposed the formula must be changed accordingly. With about 70% decomposition the formula for a bi-molecular reaction was shewn to apply. The mono-molecular formula did not fit the results obtained by Nemec and Duchon, who therefore devised the modification above, in which they substituted $\sqrt{t}$ for t. Even this modification however, did not fit the curves obtained by the present writer. In an investigation into this point it was found that if K were kept constant the experimental t was greater than the theoretical t by a number which increased by the power of x, such that, if this number were called 1 the formula became

$$K = \frac{1}{t - 1^x} \log \frac{a}{a - x} .$$

This value l is dependent on the curve itself and may be deduced by means of a quadratic equation obtained from two values of x, the one double the other. (Since K is constant

$$\frac{1}{t - l^x} \log \frac{a}{a - x} = \frac{1}{t - l^{2x}} \log \frac{a}{a - 2x}).$$

This formula fitted some of the curves very satisfactorily, but not others. This appeared to be due to the fact that variations in the speed of shaking affected the shape of the curve such that with two experiments identical except for the rate of shaking, two entirely different values of K were obtained, the sharper curve giving the higher value. Since, however, the reactions were never allowed to go to completion (as, keeping the practical end in view, this would require too long a time) it was impossible to tell to which category the reaction was related, i. e., whether to apply the bi-molecular or other formula.

The fourth and fifth methods have already been fully discussed in the review of literature. The fifth, the calculation of a set of factors to correct readings in which the depressive effect of one or other of the reacting substances is in evidence, as suggested by Rhine, appears to afford a satisfactory solution to all these difficulties, is convenient to apply and enables one to use the simplest and least troublesome reading, namely the volume of oxygen liberated after a given time.

The adverse effect of acidity on the catalase reaction has been stressed. The use of an excess of $CaCO_3$ both in the ground material and in the peroxide is a convenient means to assure practically the optimum pH; but since the peroxide decomposes spontaneously rather rapidly it is inadvisable to neutralize the acidity of the peroxide till just before the experiment.

Since it has been concluded that an arbitrary time limit reading corrected by a suitable set of factors affords the most practical measure of catalase activity, it is necessary to work with a carefully standardized peroxide solution.

Among a series of preliminary experiments carried out by the author, are some which throw some light on these points. In these the stated quantity of

seeds was ground with a pinch of $CaCO_3$ and sand and with sufficient water to form a paste. Grinding was continued till a smooth paste had been secured, when sufficient water was added in successive portions to make up a total of 100 cc. water added. This was allowed to extract the catalase for a given length of time. Four portions, each sufficient to provide an aliquot of 10 cc. were then withdrawn and centrifuged for varying lengths of time, the first to be tested for 15 minutes and each subsequent one an additional 15 minutes. It was found that 15 minutes centrifuging at 1000 revolutions per minute was often sufficient to bring the aliquot to a stable condition of activity but that 30 minutes centrifuging would always bring this about. This is illustrated in Table II.

TABLE II Cc O_2 liberated after unit time by samples centrifuged for different periods.

Series.	Expt.	Centrifuged. Minuted. 15	30	45	60
22	1a	5.2	5.3	5.3	5.4
22	1b	8.2	6.2	6.3	6.3
22	2a	2.7	2.8	3.0	2.7
22	2b	4.4	4.2	4.2	-
22	3a	4.4	4.3	4.1	4.2
22	3b	6.4	5.9	5.8	5.8
24	1	7.8	7.1	7.0	6.8
24	4	7.3	6.8	6.7	6.8

Using 10 cc. of a peroxide solution having 134.4 cc. available O_2, the results shewn in Table III were obtained. In this series a given number of seeds of different 100-kernel weights were used, the proceedure outlined above being followed.

TABLE III (Series 24) 50 kernels of wheat of different weights compared.

Sample No.	Weight	Cc O_2 after 475 seconds	Cc O_2 per unit weight
4	1.90	7.18	3.78
1	2.04	7.38	3.62
3	2.19	8.66	3.95
5	2.19	9.24	4.22
2	2.37	10.76	4.54
6	2.41	11.68	4.85

At the time this series was carried out certain points which have come to light in the preparation of the foregoing review were not fully appreciated, hence samples No's. 2 and 3 which were done at a different time to No's. 4, 5, and 6, cannot be directly compared with them. Nevertheless, the general depressive effect of excess of peroxide is clearly shewn. All the samples were from the same bulk sample and were selected according to size. Each result is the average of the closest two or three of at least three runs.

In another series (Series 23) different numbers of seeds of the same kernel-weight were used. The peroxide strength was approximately 100 cc. available O_2 ~~and~~ 10 cc. of solution. Table IV shews the results of this series.

TABLE IV (Series 23) Varied numbers of kernels of the same kernel weight compared. I.

Sample No.	No. of seeds.	Weight.	Cc O_2 after 275 seconds.	Cc O_2 per unit No. or weight.
1	50	2.19	7.55	1.51
2	60	2.63	10.71	1.79
3	70	3.07	11.88	1.70
4	80	3.50	15.13	1.89
5	90	3.94	16.56	1.84

Series 25 was similar to the above, except that different numbers of kernels were used and a reaction chamber of different shape was employed; also the peroxide was standardized to exactly 100 cc. available O_2 in 10 cc.

Table V gives the results of this series:-

TABLE V (Series 25) Varied numbers of kernels of the same kernel weight compared. II.

Sample No.	No. of seeds.	Weight.	Cc O_2 after 275 seconds.	Cc O_2 per unit No. or weight.
1	30	1.28	3.52	1.17
2	40	1.71	5.70	1.43
3	50	2.14	9.13	1.83
4	60	2.57	10.95	1.82
5	70	3.00	13.16	1.88

These two series also shew the depressive effect of a preponderance of peroxide. In Series 25 there is seen to be a sudden jump in activity between samples 2 and 3. Comparison of samples 3, 4 and 5 of this series with samples 1, 2 and 3 of series 23 also shews a relatively increased activity for the three samples of Series 25 under consideration. These samples were given much more vigorous shaking, namely four excursions per second as against one-half per second for the remainder of the samples of these two series. We must conclude therefore that speed of shaking does influence the speed of the catalase reaction when such widely divergent speeds are compared. That this effect is greater during the earlier stages of the reaction is shewn in Table VI in which the activities of two samples are compared after 150 seconds and 600 seconds for different shaking speeds.

TABLE VI Shaking speeds compared.

Sample No.	Shaking speed.	Oxygen liberated. 150 secs.	600 secs.	Relative activity. 150 secs.	600 secs.
25/1	½	2.1	5.5	64	95
25/1a	4	3.3	5.8	100	100
25/2	½	3.9	7.8	85	95
25/2a	4	4.6	8.2	100	100

In order to get rid of undue depressive effect on account of excess of peroxide therefore, and also to bring a greater proportion of the later stages of the reaction within the time limit set to minimize the effect of variations in shaking speed, the proponderance of peroxide used should be considerably lessened. In the rest of the experiments here reported therefore, 5 cc. of H_2O_2 standardized to exactly 10-volume strength have been used. In testing the peroxide, 5 cc. were diluted to 50 cc., 5cc. of which were then tested with an excess of catalase material.

Heinicke (10) found that on letting an apple-leaf catalase preparation stand, its activity increased up to one hour by which time it had reached a stable condition. In the author's preliminary experiments, the time that elapsed from the first moment of contact between the finely crushed seeds and the water till centrifuging was commenced was 18½ minutes. In order to determine whether in this matter a seed catalase prepara-

Effect of Time of Maceration on Catalase Activity.

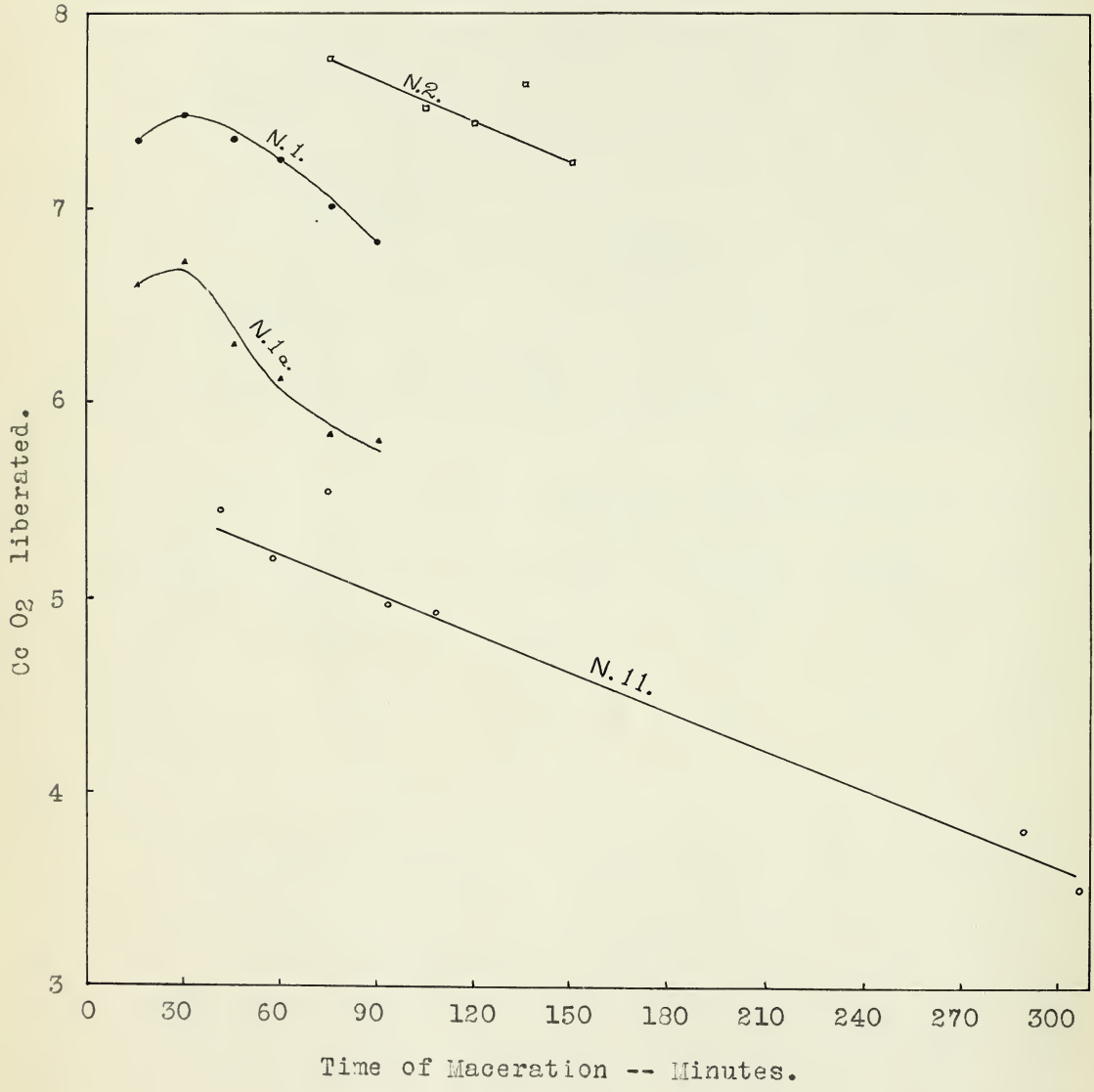


Fig. 8.

tion would behave in the same way as the apple-leaf preparation used by Heinicke, three series, N1, N1a and N2 were carried out.

In each series 2 grams of seed were ground to a paste for 5 minutes, washed into a flask calibrated to 115 cc. and shaken at intervals. In N1 and N1a sufficient preparation was poured off (after thorough shaking) into centrifuge tubes after 15, 30, 45, 60, 75 and 90 minutes, to provide aliquots of 10 cc. from each tube, which were tested immediately after centrifuging; in N2 the aliquots were poured off after 75, 90, 105, 120, 135 and 150 minutes. Results are shewn graphically in Fig. 8.

While the three series shew very different activities for their respective preparations, N1 and N1a shew a sharp initial rise in activity followed by a rapid falling off, while N2 shews that this fall continues up to 150 minutes. In N2, the 135-minute result is out of line with the rest and the 90-minute test was spoilt, but the general trend is sufficiently evident.

This was further confirmed in a later series, N 11, in which aliquots were withdrawn by pipette and tested entire without centrifuging. The results of this series are shewn in graph N 11 of Fig. 8.

In this connection it is interesting to note as in Table II, that the catalase activity of centrifuged samples did not diminish after standing up to 60 minutes, but diminished activity was only observed when maceration was continued and this, whether the samples were centrifuged before testing or tested entire. This seems rather obscure, but may indicate that the soluble (β) catalase may, after solution, gradually become absorbed by the solid particles.

It is apparent that a wheat-seed catalase preparation does not behave in this connection like the apple-leaf preparation mentioned above, but deteriorates rapidly. Further, closely comparative results seem hard to secure, since all the above samples were prepared in exactly the same manner and with the same material, except that in N1 $CaCO_3$ was omitted in the preparation of the paste, which, however, should tend, if anything, to decrease catalase activity instead of the reverse as was the case. Again, frothing of the preparation makes it very difficult to pipette aliquots accurately.

For these reasons it was decided to prepare samples by a dry method. To this end a number of tests were made on samples prepared in different ways. That which was finally adopted and used throughout the last section of this paper was the following:-

From 200 to 300 seeds (roughly) taken from different parts of the pile of seeds to be tested were placed in a flat dish to facilitate inspection. From them was removed all extraneous matter, also such seeds as were too badly injured, from the point of view of the seed analyst, to be considered suitable for setting in a germination test. This sample was then coarsely ground in a coffee grinder to particles of about the size of timothy seed. After careful mixing 1 gram was weighed and placed in a mortar with $\frac{1}{2}$ gram $CaCO_3$ and $\frac{1}{2}$ gram washed sand. This mixture was then ground by hand for exactly $2\frac{1}{4}$ minutes.

In a series of tests (N 11 a) to determine the optimum time of grinding using exactly the above method, except for the point to be determined, the results shewn in Table VII were obtained.

TABLE VII Effect of grinding catalase preparation for different lengths of time.

Time of grinding.	Cc O_2 liberated.
2 mins.	7.65
$2\frac{1}{2}$ "	7.49
3 "	7.65
4 "	7.05
6 "	5.15
8 "	5.09
10 "	3.72

This shews a rapid falling off of activity due to excessive grinding, but little difference between two and three minutes. A fraction more than two minutes grinding was therefore adopted as just stated.

This finely ground sample was then mixed with a spatula, and from it were weighed the portions to be used for test. The test sample was transferred to one arm of the Y-shaped reaction vessel and 10 cc. H_2O added, about $\frac{1}{2}$ cc. being added first and the vessel shaken till a fine suspension was secured, after which

Catalase Units corresponding to Cc O_2 liberated.

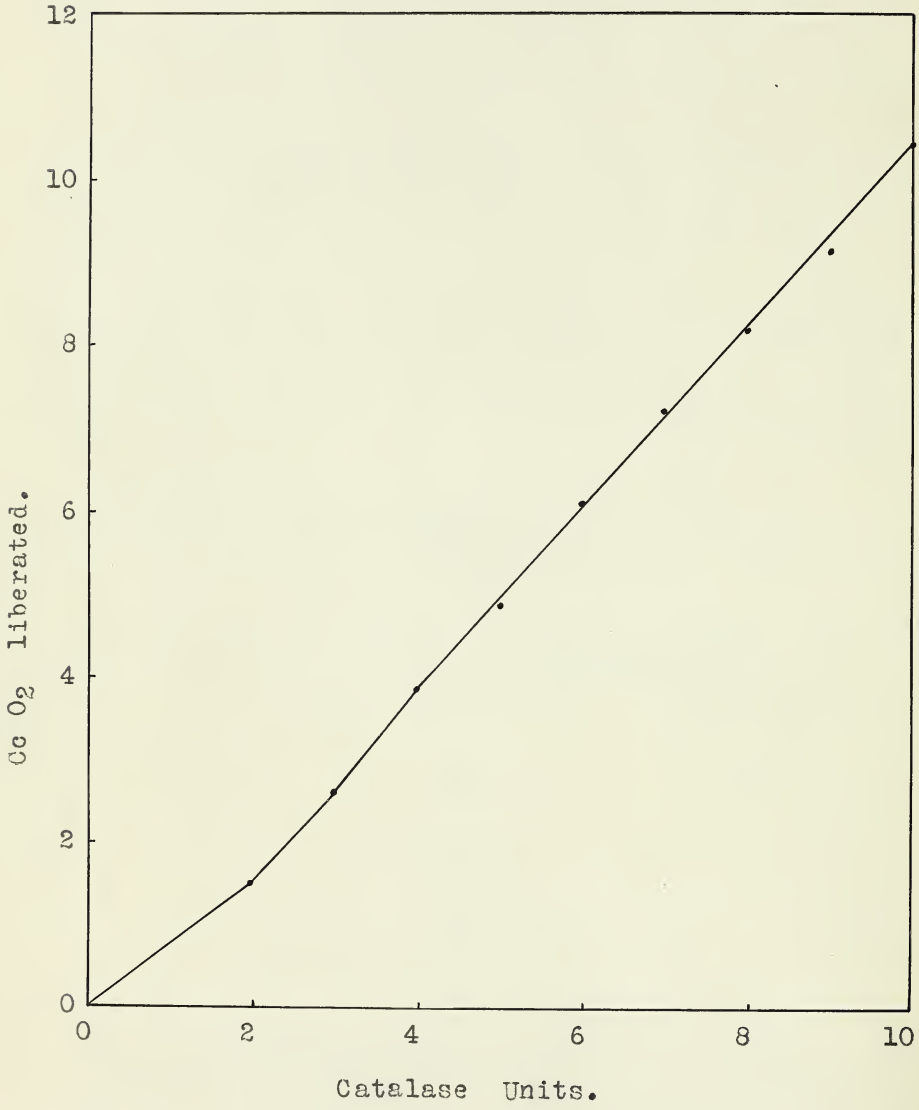


Fig. 9.

the balance of the water was added. This procedure was found to be important, as otherwise the powder caked, preventing the peroxide from penetrating. In the other arm were placed 5 cc. of standardized H_2O_2 and the vessel was connected to the apparatus. It was noticed however that a rapid absorption of air took place after attachment and before the liquids were mixed, amounting to about ½ cc. This was found to be complete in about 5 minutes, consequently this time was allowed to elapse before adjusting the level in the burette to zero, although the column of water in the graduated part was adjusted to reach nearly to the top during this process to avoid extra absorption due to pressure.

In order that the catalase reactions of samples shewing marked differences in activity might be compared on an equitable basis, it was necessary, as has already been discussed, to prepare a set of correction factors or other means to correct for the depressive effect of one or other of the reagents. In series N 8 samples of catalase preparation varying in weight from 0.0750 gr. to 0.3750 gr. were tested in triplicate with results indicated in Table VIII.

TABLE VIII Catalase units corresponding to Cc O_2 liberated.

Weight of sample.	Catalase units.	Cc O_2 liberated.
0.0750	2	1.49
0.1125	3	2.60
0.1500	4	3.89
0.1875	5	4.85
0.2250	6	6.11
0.2625	7	7.23
0.3000	8	8.20
0.3375	9	9.17
0.3750	10	10.43

This was plotted on the graph Fig. 9. and in all subsequent work, except where specifically mentioned the somewhat arbitrary catalase unit has been read from it and used as a measure of the catalase activity of the sample.

Samples prepared in this way were found in repeated tests to suffer no depreciation in activity for several days.

The Catalase Reaction Applied to the Study of Seed Viability.

In the papers of Crocker and Harrington (4) and Davis (6) who failed to find the correlation between catalase activity and germination claimed by Nemec and Duchon, no mention is made as to whether the experiments of the latter were exactly duplicated. Apparently, according to the writings of these and other authors on catalase activity in **relation** to seed germination, (except Nemec and Duchon, and De Vilmorin et Cazaubon) the catalase activity measured is the total activity, whereas that which Nemec and Duchon found to be correlated with germination was the total activity less the residual activity exhibited when the same material had been treated for 20 minutes at 100° C on the water-bath. Davis however did not base his method on the direct comparison of the total catalase activity of seeds but rather on the relation exhibited between the total activity of the seed before and after treatment of the whole seed by a soak of from one to several hours at a temperature of from 54° C to 30° C.

It would appear at first sight that there is a connection between this treatment and that of Nemec and Duchon but the results obtained are exactly the opposite in the two cases. With his treatment Davis finds that the ratio treated: untreated approximates unity in the case of seed of high viability and is less than unity in the reverse case, while the results of Nemec and Duchon, though the authors did not express them in that form, would shew approximation of the ratio to unity with seed of low viability but much less than unity in the reverse case. The explanation appears to be in the more drastic nature of the treatment in the latter case which, one would deduce from their paper, would reduce the treated seed to a condition of shewing ' catalase activity not related to viability ' the subtraction of which from the total catalase activity would give the ' catalase activity presumably related to viability ' . In the case of Davis' treatment however, since the seed was still whole and thus protected to a certain extent against catalase disintegration and since further, the treatment was not drastic enough to destroy the catalase except in the case of seeds of low viability, these would be the only kind to sustain loss in catalase, and that roughly to the extent of what in Nemec and Duchon's experiments might be considered ' catalase presumably related to viability ' .

Both methods are claimed by their authors to give figures indicative of the viability of the seed, though the results reported by Davis do not show very much more than that a sample has low or high viability; i.e. there appears to have been no attempt made to connect a given catalase result with a definite percentage of germination.

Materials and Methods.

In this section the methods outlined in the previous section were used in conjunction with the constant temperature cabinet previously described.

Samples of wheat of the 1920 and 1921 crops which had been used in frost studies and represented many different stages of maturity and frost injury, and of which the full history was known, were kindly supplied by the Department of Field Crops, University of Alberta. In addition to these there were some samples which had passed through the laboratory, presenting different degrees of weathering and other injury. Only samples which were thoroughly after-ripened by prolonged storage were used in order that difficulties of interpretation which might result from the use of dormant seed might be avoided.

Experimental.

Owing to the fact that the dry method appeared to give the best results and that it had been developed to the stage at which it might be relied upon, it was felt best first to check the method outlined by Nemec and Duchon.

Series N 9 a. The samples used in this series with their corresponding germinations and other data are given in Table IX.

TABLE IX Samples used in Series N 9 a.

Sample.	Germ. 12 days.	Remarks.	Degrees of frost to which subjected.
Plot D/20	14	Mealy dough	1, 2, 4, 7, 13.
65-1997	22	Badly frosted & weathered	Exact degree unknown
D-11-21	34	Clear green	14.
B-11-21	36	Yellow kernel	6.5, 14.
C-11-21	65	Green to yellow	6.5, 14.

TABLE IX ctd.

Sample.	Germ. 12 days.	Remarks.	Degrees of frost to which subjected.
C-12-21	71	Pale yellow	6.5, 14.
65-6501	80	Weathered, plump.	Frosted, but exact degree unknown.
C-10-21	88	Green to yellow	6.5.
#1	95	(1926 reg. crop, (very high quality (but maintained at (R.H. 71% several (months.	0.
C-1-21	99	Whitish (very immature)	0.

(Notes on all samples except 65-6501, 65-1997, and #1 taken from notes made by the University of Alberta at time of harvesting.)

Catalase tests were made on these samples, both on the treated and untreated ground material, the treatment consisting of placing the weighed experimental material for 20 minutes in an oven containing a dish of water and regulated to 98°C, this being the nearest approach to Nemec and Duchon's treatment on the water-bath that it was possible to make under the circumstances. (The boiling-point of water at the elevation of Calgary is approximately 98°C.)

Three replicates were made on each of the treated and untreated samples. Where one replicate was divergent from the other two, the two close ones were used; where there was a moderate spread between the three, the average of all three; but where there was no close agreement, a fresh sample was prepared and tested. These remarks apply also to Series N 13, reported later.

Results of these determinations are given in Table X.

TABLE X Series N 9 a. Catalase determinations.

Sample.	Germ.	Cc. O2 liberated. Untreated.	Treated.	Nemec and Duchon figure	Cc. O2 ratio.
Plot D/20	14	6.43	3.16	5.75	.491
65-1997	22	5.77	2.73	3.04	.473
D-11-21	34	8.74	5.68	5.36	.650
B-11-21	36	6.32	3.94	2.38	.623
C-11-21	65	6.60	4.14	4.31	.627
C-12-21	71	6.24	3.44	4.90	.551
65-6501	80	6.36	4.29	2.07	.675
C-10-21	88	7.40	4.62	4.87	.624
#1	95	7.38	4.68	2.70	.634
C-1-21	99	10.15	7.18	5.19	.708

In the fifth column is the figure Nemec and Duchon use for their expression of seed viability. This figure has been obtained by subtracting the number of cc. of oxygen liberated by the treated sample from that liberated by the untreated; but note that on account of the great activity in all samples except 65-1997, B-11-21, 65-6501 and #1, in which 0.2625 grams were used, the size of the sample had to be reduced to 0.1500 grams, hence the figure obtained by subtraction had to be multiplied by 7/4. There appears to be no correlation between this figure and the germination of the seed.

Since these samples shew very uneven catalase activity, it was thought that perhaps if the ratio of the activity of the treated to that of the untreated were compared, a correlation might be revealed. In the sixth column these ratios are tabulated but again no correlation is apparent.

If these samples are arranged in order of their maturity as indicated in Table IX there is seen to be a striking inverse correlation between catalase activity and maturity. This is shewn in Table XI, the factor 7/4 being introduced as before to bring all samples to an even weight basis.

TABLE XI Maturity of seed as affecting catalase activity.

Sample.	Catalase activity. Untreated.	Treated.	
C-1-21	17.76	12.57	
D-11-21	15.50	9.94	
C-10-21	12.95	8.09	
C-11-21	11.55	7.25	
C-12-21	10.92	6.02	
Plot D/20	11.28	5.53	
B-11-21	6.32	3.94	) Not much difference
65-1997	5.77	2.73	) between maturity of
65-6501	6.56	4.29	) these samples, but
#1	7.38	4.68	) to best judgment as arranged.

Nemec and Duchon worked with samples of presumably the same degree of maturity, but which had lost their vitality through age. The effect of seed exhibiting increased catalase activity with immaturity may be to mask the effect of the treatment suggested by these authors.

It occurred to the present writer that possibly the treatment at 98°C for 20 minutes did not in fact reduce the test sample to a condition of exhibiting only thermo-stable catalase activity and to question whether there was such a thermo-stable residue. In Series N 12 this point was investigated.

In the series just discussed, the figures used have been on the basis of cc. O_2 liberated and not corrected to catalase units, since this is the basis used by Nemec and Duchon. The conclusions are unaltered however when catalase units are used as the basis. In Series N 12 and N 13 catalase units have been used throughout.

Series N 12. In this series the test samples, all prepared from one bulk sample, were heated for 10, 20, 30, 45, 60, 75, and 90 minutes respectively. All tests were made in quadruplicate, two replicates being from one grind and two from another for each treated sample, and from each grind a check untreated test was also made. Results were then calculated to the basis of the highest set of checks, since absolute uniformity

Series N 12.

Effect of Heat on Catalase Activity.

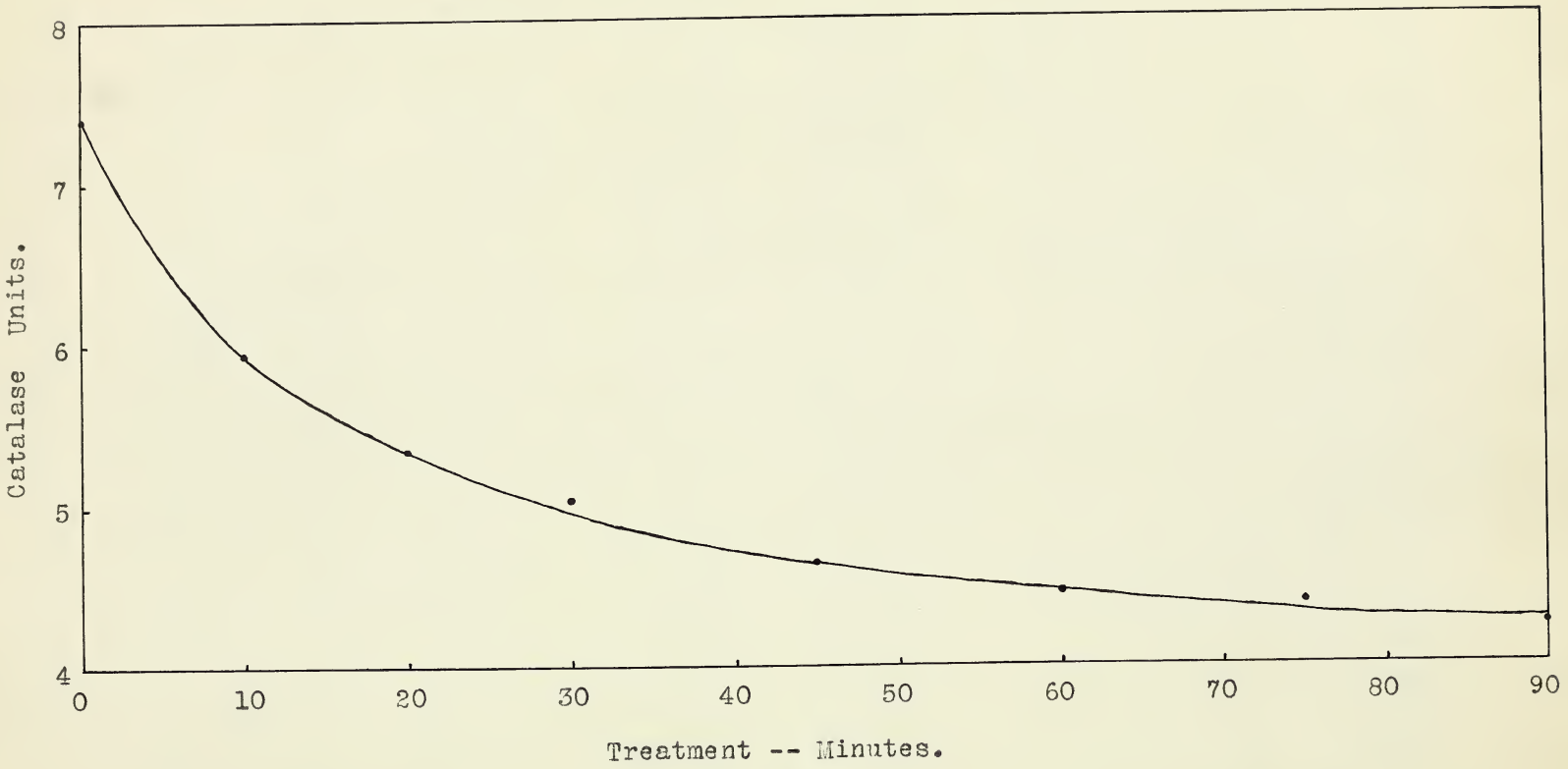


Fig. 10.

between the different grinds was not obtained. The graph, Fig. 10. shews the results obtained in this series.

It is clear from this graph that 20 minutes heating by no means destroys all the thermo-labile catalase but that the curve becomes very nearly horizontal after treatment for 90 minutes, at which point there is a very considerable residue of catalase which must be considered as thermo-stable.

It seemed possible that the proportion of truly thermo-stable catalase in the seed might bear a relation to the viability of the seed. Series N 13 was therefore carried out, on the same plan as N 9 a, but in which all treated test samples were heated for 90 minutes at 98° C.

Series N 13. The samples used in this series are shewn in Table XII. Some of these are the same as those used in N 9 a, but are repeated here for convenience.

TABLE XII. Samples used in series N 13.

Sample.	Germ. 12 days.	Remarks.	Degrees of frost to which subjected.
D-16-20	8	Mealy dough-wet.	13
65-1997	22	Badly frosted & weathered.	Exact degree unknown.
D-11-21	34	Clear green.	14
B-11-21	36	Yellow kernel.	6.5, 14.
C-11-21	65	Green to yellow.	6.5, 14.
C-12-21	71	Pale yellow.	6.5, 14.
65-6756B	73	Weathered.	(Frosted, but exact
65-6756A	77		(degree unknown.
65-6501	80	Weathered, Plump.	" " " "
65-6883	87	Uneven maturity.	Some frost.
#1	95	(1926 Reg. Crop, very high quality, but maintained at R.H. 71% several months.	0.
D-7-20	97	Medium dough.	4.
A-16-20	100	Hard.	0.

(Notes on those samples where exact degree of frost given (except #1) made by the University of Alberta at time of harvesting.)

Series N 13.

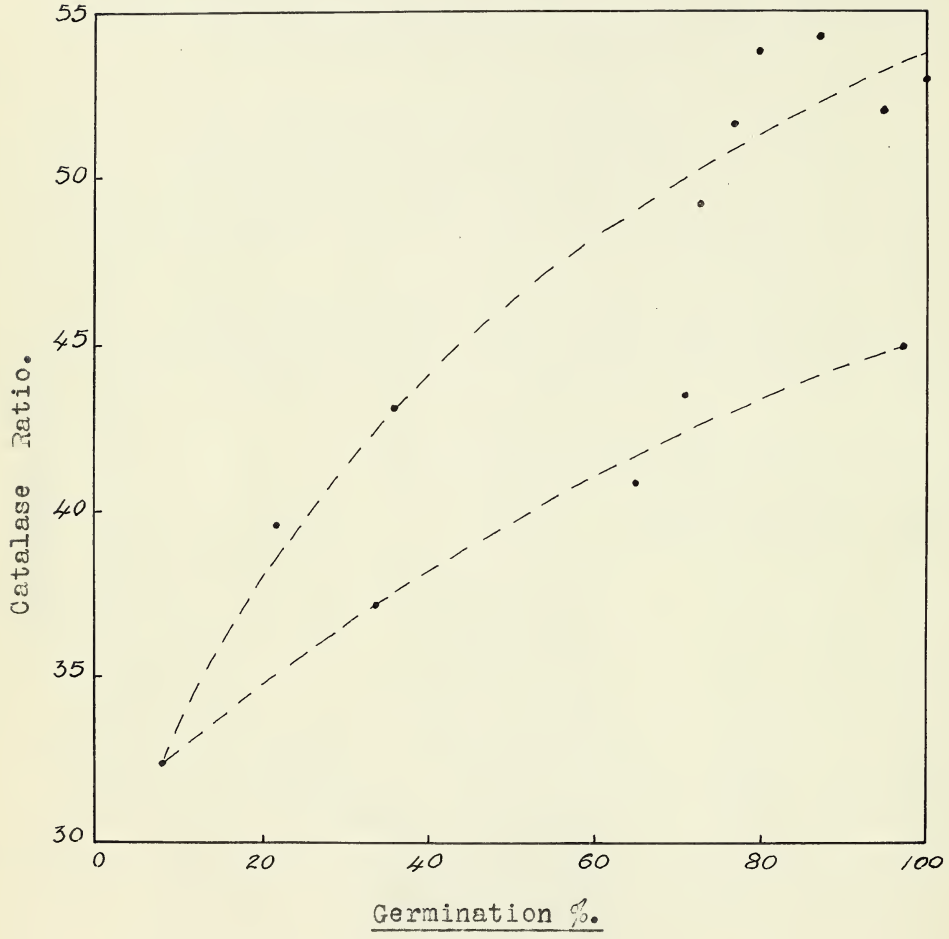


Fig. 11.

Results in this series have been calculated to catalase units and on account of the great differences in individual catalase activities, have been expressed as the ratio of thermo-stable to total catalase in the form of a percentage. These are shewn graphically in Fig. 11.

While there is not the close correspondence it had been hoped to secure between this catalase ratio and the germination of the seed, yet on the whole, the samples of low viability shew markedly lower ratios than those of high viability.

However, the points on this graph are seen to lie more or less in two zones, a lower and an upper, as indicated by the dotted curves. It we take the samples represented by the points near each curve and tabulate them separately as in Table XIII an interesting and possibly significant point becomes evident.

TABLE XIII Samples shewing relatively high and low catalase ratios, tabulated separately.

Samples shewing high catalase ratios.

Sample.	Germ.	Ratio.	Total Catalase.
D-16-20	8	32.4	8.01
65-1997	22	39.6	6.62
B-11-21	36	43.1	5.75
65-6756B	73	49.3	6.80
65-6756A	77	51.7	7.56
65-6501	80	53.9	6.21
65-6883	87	54.3	8.20
#1	95	52.1	7.78
A-16-20	100	55.0	5.83

Samples shewing low catalase ratios.

D-16-20	8	32.4	8.01
D-11-21	34	37.2	14.98
C-11-21	65	40.8	10.94
C-12-21	71	43.5	10.55
D-7-20	97	44.9	14.51

In the last column the total catalase activity of each sample is shewn. One sample (D-16-20) finds a place in both curves. Now all the samples in the group shewing low catalase ratios, except D-16-20, were such as

exhibited such high total activity that the amount of material tested had to be reduced to 0.1500 gms. in order that the burette might accommodate the oxygen liberated. It has previously been shewn that high total catalase activity is associated with immaturity of the seed at the time of harvesting, hence this is an important point to be considered in the interpretation of results.

Discussion.

This method appears to be capable of being developed into a useful accessory to the germination test, but a considerable amount of further study is required.

Not all samples may lose their thermo-labile catalase in the 90-minute heating period, in which case those which gave an unduly high ratio might be brought more into line with the others. (The sample used to test the loss of thermo-labile catalase was a well matured frost-free sample of good quality, of which there was sufficient to make all the tests required.)

Relative degrees of immaturity of the seed introduce a complication which may require the introduction of a correction factor in order that all samples may be compared on an equal basis.

While the dry method developed is not entirely reliable for the direct comparison of catalase activity between samples, this is not of much moment when the comparison is to be made between treated and untreated test samples taken from the same grind, for replicates of any one grind shew quite close correspondence. Where direct comparison between samples is required this may be done by preparing two or more grinds from each sample.

Summary.

That the germination method of testing seed viability, requires a considerable time before the results can be known, is apt at times to be a serious hindrance to trade.

This has led investigators to turn their attention to finding an index of seed viability other than that of germination. Among the methods proposed, that making use of the catalase reaction has been chosen for study. Literature on these methods, and on the catalase reaction in general has been reviewed and summarized.

This study has been confined to one kind of seed, viz; spring wheat.

Two methods of preparing the experimental sample

have been tried out, a wet and a dry.

In the wet method, the time of maceration adversely affected catalase activity, but centrifuged aliquots maintained their activity unimpaired up to one hour. Centrifuging for thirty minutes at 1000 revolutions was necessary to bring all samples to a stable condition of activity.

A dry method was finally adopted as more suitable. The catalase activity, however, was shewn to be inversely proportional to the time of grinding, which necessitated the adoption of a standard time for grinding.

The depressive effect of excess of peroxide reported by other workers was observed.

The speed of shaking during the reaction was found to affect the activity, but this was more marked in the earlier stages of the reaction, and with a great difference in shaking speeds.

A graph in which oxygen liberated is plotted against catalase units was prepared and used in correcting for the depressive effect of excess of one or other of the reacting substances.

An attempt was made to duplicate Nemec & Duchon's work, but without securing the same results. It was observed, however, that not all the thermo-labile catalase was destroyed by heating for 20 minutes at the termperature of boiling water, and a series of experiments shewed that this destruction did not occur until after about 90 minutes of such treatment.

A fresh series of experiments in which samples of different viability were tested before and after this 90 minute treatment shewed a certain measure of correlation between the ratio thermo-stable: total catalase and viability. A graph on which the catalase ratio was plotted against the germination shewed the points plotted to be arranged more or less in two zones, in one of which the catalase ratios were proportionately much lower for their corresponding germinations than in the other, but in each of which there was a fairly good relationship between the catalase ratio and germination. The lower set of ratios were shewn to be associated with samples having a considerable degree of immaturity and exhibiting high total catalase activity in consequence. The question of immaturity as affecting the catalase ratio is one requiring further study.

I have great pleasure in expressing my thanks to Dr. R. Newton, Professor of Field Crops & Plant Biochemistry, under whose guidance this work has been undertaken, and to Dr. J. W. Campbell, Professor of Mathematics, for help in the mathematical parts.

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